Loss of Class II HDAC10 Expression on Osteoclastogenesis

A THESIS

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Dedication

To my dear parents for their endless love, care and support.

Abstract

Osteoclasts differentiation is regulated by a complex series of genes. HDACs, histone deacetylases, are a family of proteins that inhibit gene expression in multiple cell types. Class II HDACs are expressed in multiple cell types and found either in the nucleus or the cytoplasm or both cellular compartments of cells. This localization suggests that they have roles in regulating cell differentiation. The Mansky lab and other have demonstrated that the class II HDACs, HDAC7 and HDAC9, act as inhibitors of osteoclast differentiation. The aim of my project was to investigate the role of HDAC10, another class II HDAC, in regulating osteoclast differentiation. Osteoclasts expressing a shRNA against HDAC10 were larger in size and demineralized more of a calcium/phosphate substrate compared to control infected cells. This data suggests that similar to HDAC7 and HDAC9, HDAC10 acts as an inhibitor of osteoclast differentiation.

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Introduction

Osteoclasts

Bones have numerous functions in the body such as mechanical support of soft tissues, being a lever for muscle action, protection of the central nervous system, maintenance of a constant ionic environment in the extracellular fluid, and housing and support of hematopoiesis. In order to maintain their integrity, bone undergoes continuous remodeling, resorption, carried out by osteoclasts, followed by bone formation by osteoblasts. In the adult skeleton, the two processes are in balance, maintaining a constant amount of bone (1). When the balance is disrupted between osteoblasts and osteoclasts, diseases such as osteoporosis, rheumatoid arthritis and Paget's disease can result. Osteoporosis is a worldwide disease that is recognized as a growing epidemic in the elderly. Osteoporotic fractures are a significant cause of morbidity and mortality in the elderly. According to O. Johnell, 2000, there were estimated 9 million new osteoporotic fractures, of which 1.6 million were fractures at the hip, 1.7 million were fractures at the forearm, and 1.4 million were

clinical vertebral fractures (2). Understanding the mechanisms that regulate osteoclast formation and function could help to develop new therapeutic solutions for bone-related disorders.

Osteoclasts are multinucleated bone-resorbing cells, and their activity has a profound impact on skeletal health. Diseases such as osteoporosis have increased osteoclast activity relative to bone formation by osteoblasts (3).

The osteoclasts are derived from the monocyte/macrophage lineage. Early osteoclast differentiation is dependent on PU.1 (transcription factor) and the MITF (microphtalmia-associated transcription factor) family of transcription factors, as well as the macrophage proliferation and survival cytokine M-CSF (macrophage colony-stimulating factor) (Fig 1). Activation of RANK (receptor activator of nuclear factor Kappa-B) by osteoblast-expressed RANK ligand (RANKL) commits the cell to the osteoclast fate, which is mediated by proteins such as AP-1 (activator protein 1) transcription factors, TRAF6 (tumor necrosis factor receptor associated factor 6), and NF-κB (nuclear factor kappa B). RANKL-stimulated osteoclastogenesis is inhibited by the RANKL decoy receptor osteoprotegerin (OPG). The initial event in development of the

resorptive capacity of the mature osteoclast is its polarization, which requires c-Src (proto-oncogene tyrosine-protein kinase Src) and the αvβ3 integrin. Once polarized, the osteoclast mobilizes the mineralized component of bone. Bone mobilization is achieved through the acidifying molecules, carbonic anhydrase II (CAII), H^+ATPase (H+Adenylpyrophosphatase) and a charge-coupled Cl^- channel. Cathepsin K mediates bone organic matrix degradation (3).

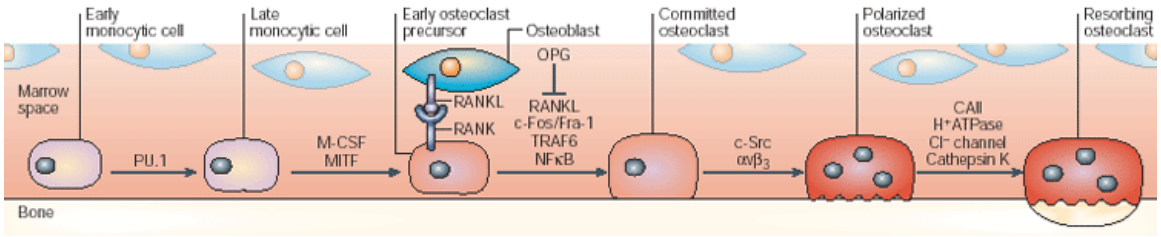

Figure 1: Regulation of osteoclasts formation and function (3)

Histone Deacetylases (HDACs)

Histones are highly alkaline proteins found in eukaryotic cell nuclei that package the DNA (deoxyribonucleic acid) into their structural units called nucleosomes. DNA is in its condensed form called heterochromatin when it is condensed around histones. In this form DNA is not available for transcription. When histones are acetylated by histone acetyltransferases (HATs), positive charges

from acetyl group allows DNA to disassociate from histones, which causes DNA to become less tightly wound (euchromatin) and allowing the DNA to be available for transcription. Histone deacytylases (HDACs) remove acetyl groups from DNA complex, which causes the condensation of the DNA around histones, and as a result negatively regulates gene expression. (4)

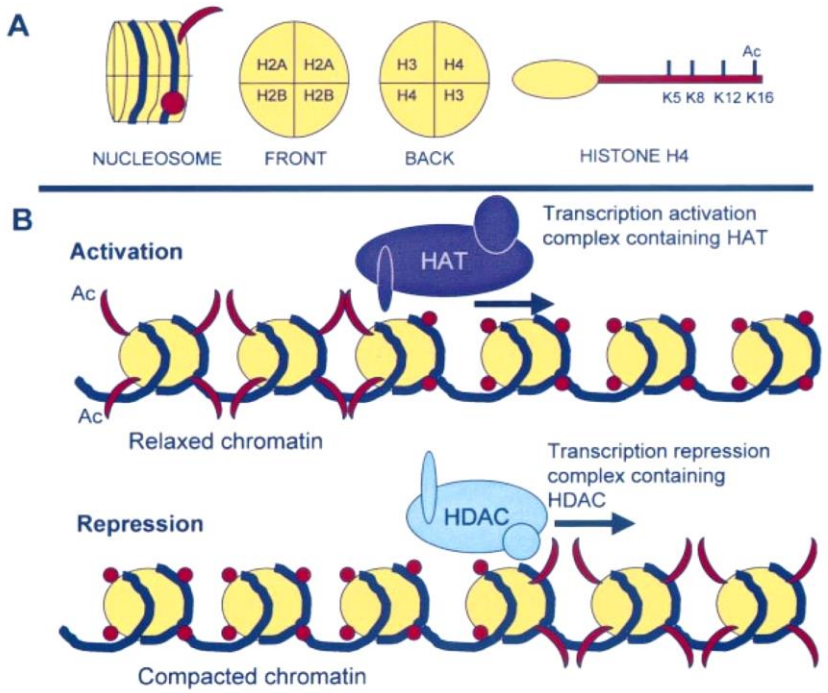

Figure 2a: Various aspects of the transcription process and its regulation by histone modification (4)

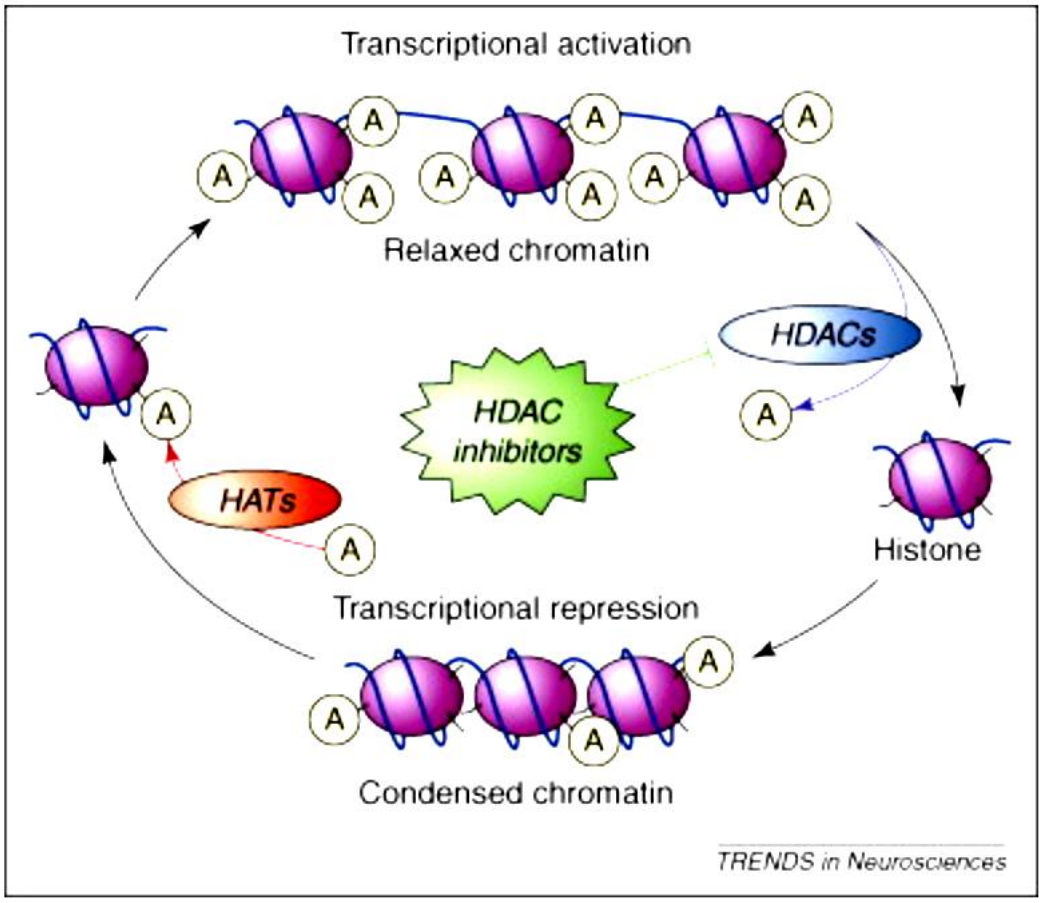

Figure 2b: Effects of HDAC inhibitors on chromatin remodeling (15)

There are two protein families, which have HDAC activity: SIR2 (Sirtulin 2) family of NAD+-dependent (Nicotinamide adenine dinucleotide-dependent) HDACs, and the classical HDAC family. Members of the classical HDAC family consist of two different classes, termed Class I and Class II. The Class I HDACs (HDAC1, 2, 3 and 8) are most closely related to the yeast (*Saccharomyces cereisiae*) transcriptional regulator RPD3. Class II HDACs (HDAC 4,

5, 6, 7, 9 and 10) share domains with similarity to HDAC1, another deacetylase found in yeast. Class I HDACs are mostly found in the nucleus, while Class II HDACs can be found in both the nucleus and the cytoplasm (4).

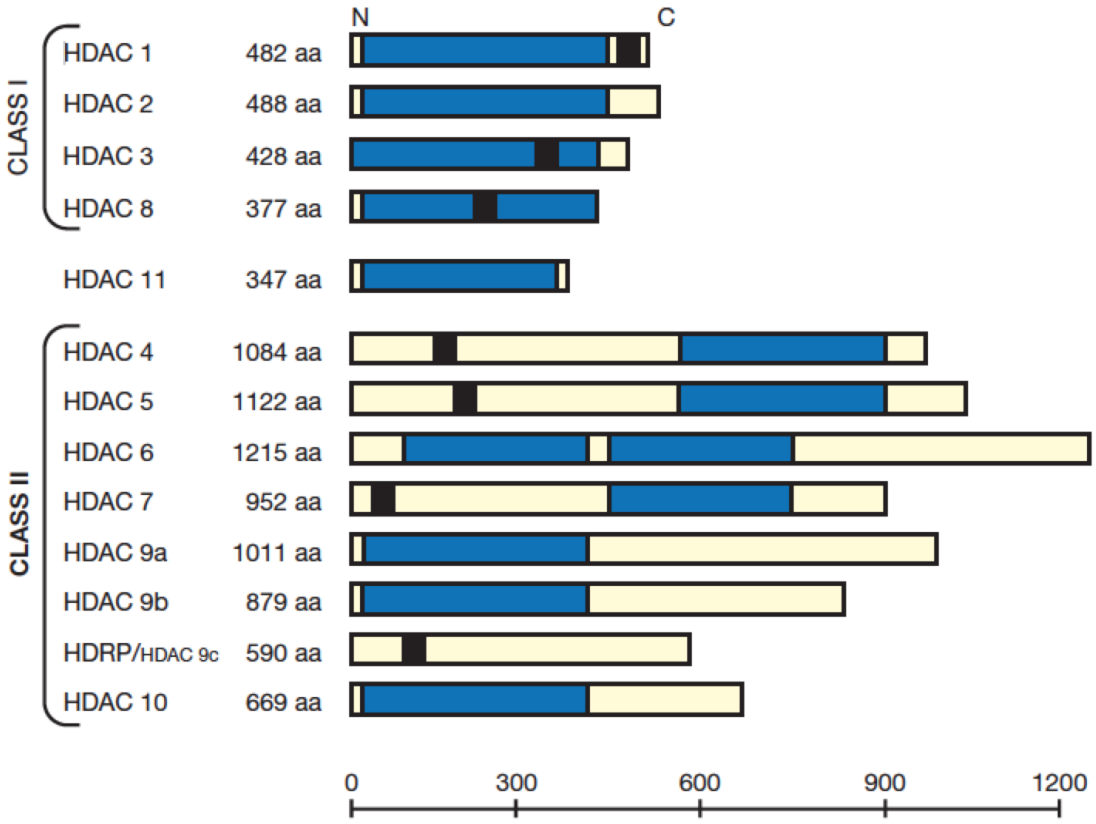

Figure 3: Schematic depiction of the different isoforms of HDAC (4)

HDACs role in Osteoclast Differentiation

There is still a lot unknown about the role of HDAC's in the osteoclast differentiation. However, there have been a few studies

investigating the role of HDACs in osteoclast differentiation. In recent research performed by the Mansky laboratory, it was reported that HDAC7 binds to and decreases the transcriptional activity of MITF, an important transcriptional factor which is essential for regulating genes required for osteoclast differentiation. The loss of HDAC7 in osteoclasts results in increased osteoclastogenesis in vitro (12). An additional study investigating HDAC7's role in regulating osteoclastogenesis by Mansky and Jensen, demonstrated that suppression of HDAC7 expression in mice results in the increase of osteoclast formation in vivo, combined with increased bone resorption and decreased bone mass (12). Recent research by Jin et al., 2015 suggested that HDAC9 also inhibits osteoclastogenesis and bone resorption. Loss of HDAC9 expression in osteoclasts results in increased bone resorption and loss of bone mass in mice (14). Furthermore, a recent research study by Dr. Mansky and Dr. Lelich demonstrated that osteoclasts deficient in HDAC4 expression produced larger and more numerous osteoclasts (5). Additionally, they demonstrated the osteoclasts with reduced HDAC4 expression demineralized more calcium/phosphate compared to control cells (5).

HDAC10

HDAC10 is relatively a new member of the expanding HDAC family (6). HDAC10 consists of N-terminal Hda1p-related putative deacetylase domain and a C-terminal leucine-rich domain that may also function as a deacetylase domain (9). A study performed by Guardiola and Tso-Pang Yao (2002) suggested that HDAC10 is most similar to HDAC6 (Fig.4). Research by Hung-Ying Kao (2002) suggests that human HDAC10 has the highest expression in the liver, kidney and spleen (8) (Fig 5), while human HDAC4 and mouse HDAC6 are most prevalent in skeletal muscle and testis, respectively (9).

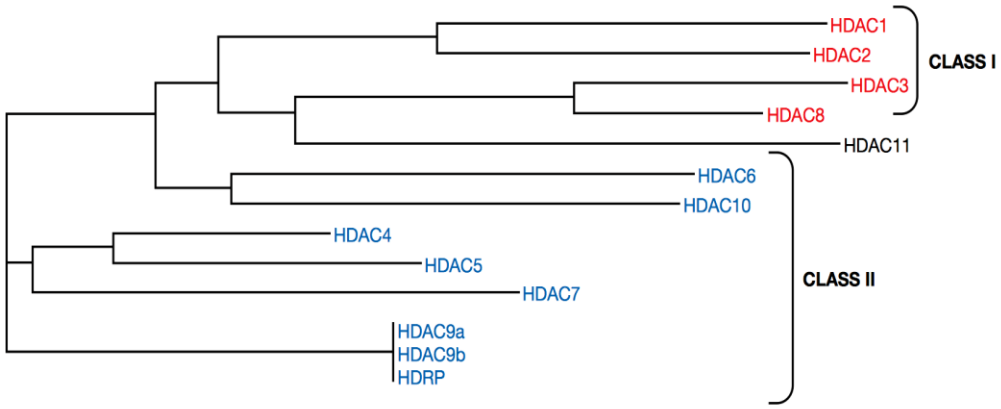

Figure 4: Evolutionary relationship between the HDACs (4)

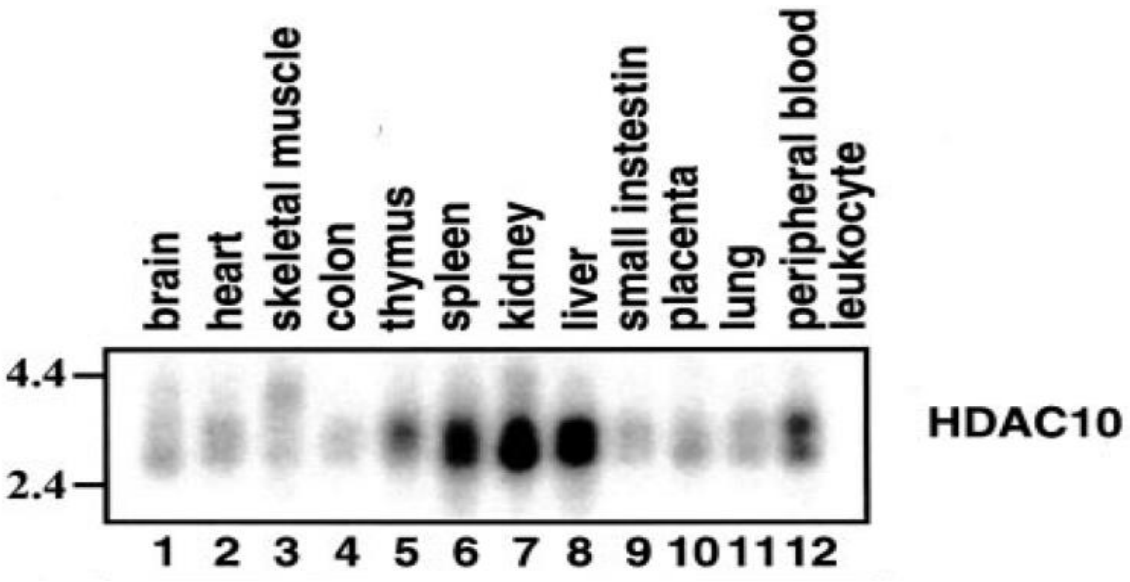

Figure 5: Tissue Distribution of HDAC10 (human HDAC10 is highly expressed in liver, spleen and kidney) (8)

Both HDAC6 and HDAC10 have a second catalytic domain, which is unique to HDAC6 and HDAC10. This two-domain configuration provides resistance to the inhibitors such as sodium butyrate and trapoxin B, which potentially inhibit deacetylase activity of other HDACs. Hence, HDAC6 and HDAC10 both have similar uncommon pharmacological and structural features. Nevertheless, HDAC10 is located in both the nucleus and cytoplasm unlike HDAC6, which is normally found only in the cytoplasm of cells including osteoclasts (4, 7). The cellular location of HDAC6 sets it apart from other class II HDACs which are commonly found in the both the

nucleus and cytoplasm. HDAC10 has been shown to be able to interact with HDAC 4, 5 and 7, which suggests that HDAC10 may act as a recruiter for other HDACs (4). Currently it is not evident what is the mechanism by which HDAC10 regulates gene expression. Additionally, the targets of HDAC10 in the cytoplasm are still unclear (7). Lastly, it still needs to be determined if HDAC10 is expressed both in the nucleus and cytoplasm of osteoclasts and does HDAC10's subcellular location change during osteoclast differentiation.

The roles that HDAC's play in osteoclastogenesis means that they have potentially therapeutic functions to treating cancers, HIV/AIDS, osteoporosis. Pharmaceutical HDAC inhibitors (HDIs) has been recently developed and tested to treat osteoporosis, Paget's disease and other bone disorders. HDAC therapy can potentially become useful in orthodontics, since orthodontic teeth movement is strongly influenced by remodeling, resorption and apposition of the bone.

Hypothesis

The hypothesis of this research project is that loss of HDAC10 expression in osteoclasts will enhance osteoclast differentiation and activity.

Specific Aims

The aims of this study: 1) to investigate the importance of HDAC 10 expression during osteoclast differentiation utilizing the Trap staining technique and 2) to determine the necessity of HDAC10 expression during osteoclast activity using the resorption plates.

Materials and Methods

Harvesting of bone marrow and primary osteoclasts

Adherent tissue was removed from the femurs and tibiae of wild type mice. The peripheral parts of the bones were cut, the bone marrow was flushed and red blood cells were lysed with red blood cell lysis buffer. The remaining cells plated on 100 mM plates and cultured overnight in osteoclast media (phenol red-free alpha-MEM

(Gibco, Grand Island, NY, USA) with 5% heat-inactivated fetal bovine serum (Hyclone), 25 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), 400 mM L-Glutamine (Invitrogen), and supplemented with 1% CMG 14-12 conditioned media containing M-CSF). The non-adherent cell population which also contained osteoclast precursor cells, was gently separated and re-plated at approximately 100,000 cells per well for resorption (24 well plate) and 200,000 cells per well for TRAP staining (12 well plate) in the presence of 1% CMG 14-12. Cells were plated on tissue culture dishes with 24 wells for each round of experiments. One plate was designated for TRAP staining and the other, a calcium phosphate coated plate, was used to analyze resorption.

Lentiviral Transfection

Lentiviral vectors that encode shRNAs (short hairpin RNA) against HDAC10 or a control shRNA were utilized to produce replication defective lentivirus according to the manufactures protocol. The control shRNA contains a scrambled sequence that is unable to anneal to any known gene. Lentivirus expressing the

shRNA was added 24 hours after plating to the cells at 37^0C in the presence of 1% CMG 14-12 conditioned media. Two distinct lentiviruses were used in this study: each of the viruses was added to 3 wells on each plate specific for reducing expression of HDAC 10. HDAC10 shRNA #1 is Open Biosystems (Lafayette, CO, USA) clone 386 while HDAC10 shRNA #2 is Open Biosystems clone 387. Identical pattern for virus addition was performed in each round of experiments. On the second day lentivirus was removed from the wells and cells were fed with 1% CMG 14-12 conditioned media and RANKL (10 ng/mL). Cells were fed every other day with RANKL and 1% CMG 14-12 conditioned media until the presence of multinuclear cells appeared in the control wells.

Trap Staining

To investigate the importance of HDAC10 expression during osteoclast differentiation as it was indicated in the first aim of the study, primary osteoclasts were washed with PBS and cells were fixed with 4%PFA (paraformaldehyde). Afterwards, the cells were stained for tartrate resistant acid phosphatase (TRAP) expression

with tartrate 5 mg, Naphtol AS-MX phosphate, 0.5 mL M,M-Dimethyl formamide, 50 mL acetic acid buffer (1 mL acetic acid, 6.8 g sodium acetate trihydrate, 11.5 g sodium tartrate in 1 L water) and 25 mg Fast Violet LB salt. The cells were photographed at a minimum 3 images per well. The software Image J version 1.49 (NIH, Bethesda, MD, USA) was used for analyzing the osteoclasts.

Resorption plates

To determine the necessity of HDAC10 expression during osteoclast activity as indicated in the second aim of the study, resorption plates were coated with calcium phosphate substrate in order to imitate bone matrix. The media was aspirated and 100 μL of 10% bleach was added to each well and incubated at room temperature for 5 minutes in order to prepare the cells on these plates. The cells were then rinsed twice with water, allowed to air dry and photographed with light microscopy. The measurements were analyzed using NIH Image J version 1.49.

Statistical Analyses:

The results were expressed as a mean +/- standard deviation. Prism version 7 (GraphPad Software, USA) was utilized to calculate the ANOVA analyses with a Tukey multiple comparison test. For all tests the adopted significance level was 5%.

Results

To understand the importance of HDAC10 expression during osteoclast differentiation, we infected osteoclasts with one of two different lentivirus constructs that expresses a shRNA against HDAC10. Osteoclasts were allowed to differentiate in the presence of M-CSF and RANKL until multinuclear cells appeared. Cells were fixed and TRAP stained, photographed and quantified using Image-J 1.49. In figure 6 (A) representative images of TRAP stain osteoclasts are shown that have either been infected with a control shRNA (control) or shRNA against HDAC 10 (HDAC10 #1 or HDAC10#2).

HDAC10 appears to act as a negative regulator of osteoclast differentiation. Suppression of HDAC10 decreases the number of multinucleated cells but increases the size of the cells.

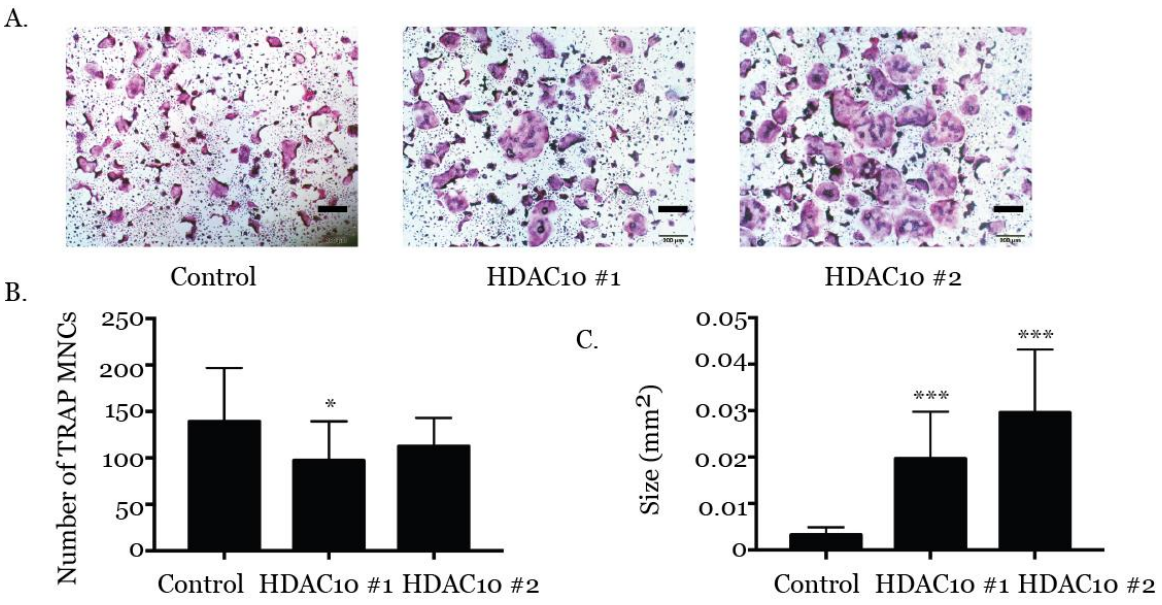

Figure 6: Suppression of the HDAC 10 by shRNA increases osteoclast differentiation

As shown in the Figure 6 (B), for one of the shRNAs (HDAC10 #1) there was a significant reduction in the number of multinucleated osteoclasts compared to the control infected cells (compare control 139, HDAC10#1 97.2 and HDAC10#2 112.4). Figure 6 shows hat the size of cells that expressed a shRNA against HDAC10 increased significantly compared to the control infected cells (compare control 0.003, HDAC10 #1 0.019 and HDAC10#2 0.029, Table 1). In a summary, as the multinucleated osteoclast cells got larger in size due to the fusion of cells and formation of giant cells, their number decreased. Both of the findings are statistically significant since the p

value is less than 0.05. Therefore, from this data we can conclude that HDAC10 appears to act as a negative regulator of osteoclast differentiation.

Table 1. Average number and size of HDAC10 shRNA expressing TRAP positive osteoclasts

	Osteoclast Number (Mean)	Osteoclast Size (μm, Mean)
Control	139 ± 57.69	0.003278 ± 0.001674
HDAC10 shRNA #1	97.28 ± 41.97	0.01972 ± 0.01008
HDAC10 shRNA#2	112.4 ±30.47	0.02967 ±0.01354

Resorption

In order to determine the effect of HDAC 10 on osteoclast activity, we infected osteoclasts with either control or shRNA against HDAC 10 as described in the materials and methods. The cells were plated on calcium phosphate substrate-coated plates. I allowed the cells to differentiate and demineralize for five days. In order to quantify osteoclast activity, I compared total pit number per well, average pit size per well and the total percent resorbed per well. Plates were treated as described in material and method,

photographed, quantified and analyzed using Image J. In both shRNA #1 and #2 the amount of pits per well increased (compare control of 47.4, HDAC10#1 104.2 and HDAC10#2 89.9), average size of pit increased (control 0.001, HDAC10#1 0.017 and HDAC10#2 0.054) and also the percent of resorbed increased compare to the control infected cells (compare control 11.5%, HDAC10#1 28.5% and HDAC10#2 34.5%) (Figure 7, Table 2).

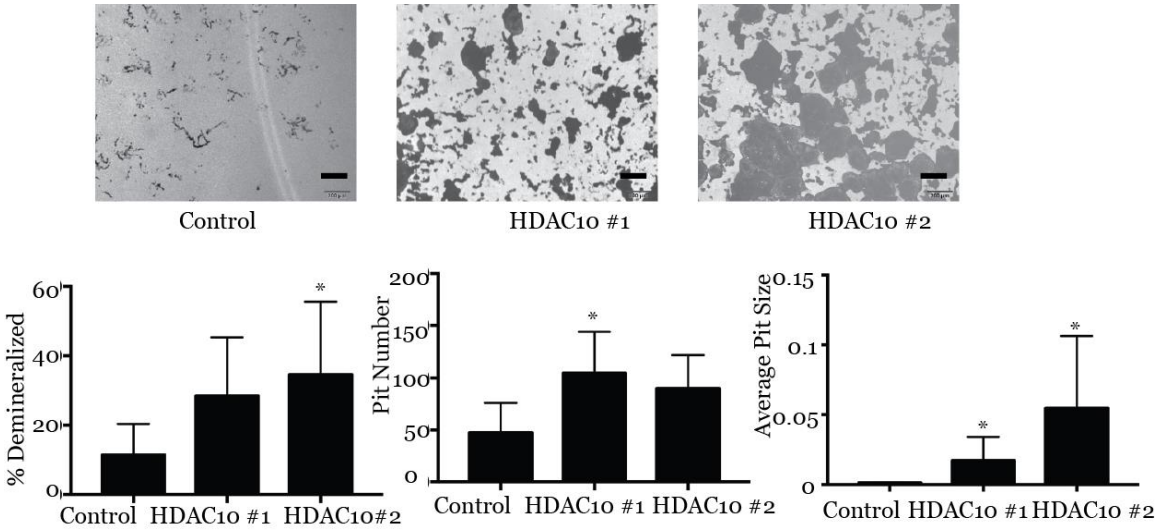

Figure 7: Suppression of HDAC 10 increases resorption

Table 2. Average Resorption Activity of HDAC10 shRNA expressing osteoclasts.

	Pit Number (Mean)	Average Pit Size (Mean)	Percent Resorbed (Mean)
Control	47.4 ± 28.75	0.00167 ± 0.0004082	11.53 ± 8.903

HDAC10 shRNA #1	104.2 ± 39.83	0.01717 ± 0.01718	28.51 ± 16.79
HDAC10 shRNA #2	89.91 ± 31.88	0.05478 ± 0.0517	34.53 ± 21.03

Discussion

HDAC10's Role in Osteoclastogenesis and Resorption

There is very limited research information available about the mechanisms by which HDAC10 regulates gene expression. This study focused on the role of HDAC10 in regulating osteoclastogenesis. As anticipated, loss of HDAC10 expression enhanced osteoclast differentiation and activity. This study showed that suppression of HDAC10 reduces number of multinucleated osteoclasts and increases the size of the cells. Osteoclasts with suppressed HDAC10 expression had significantly larger demineralized pit sizes when compared to the control cells as shown in Figure 7. Consequently, the conclusion was made that HDAC10 appears to act as a negative regulator of the osteoclast differentiation.

HDAC10 is most closely related to HDAC 6 (37% overall similarity) of the class II HDACs. However, while HDAC 6 has been shown to function as a tubulin deacetylase in osteoclasts (16), this is the first study to investigate the role of HDAC10 in osteoclasts. Unlike HDAC6, HDAC10 is thought to be expressed in both the nucleus and the cytoplasm and act as a transcriptional repressor. The mechanism by which HDAC10 represses transcription in osteoclasts is unknown.

Histone Deacetylase Inhibitors (HDACi)

HDACi has been a very rapidly growing field of research. Interest is increasing due to HDACi promising properties to treat various diseases including some types of cancers, multiple myeloma and other disorders.

Histone deacetylase inhibitors (HDACi) are a group of targeted anticancer agents. Vorinostat (suberoylanilide hydroxamic acid), which is one of the firsts of HDACi, has been recently approved by Food and Drug Administration for treating patients with cutaneous T-cell lymphoma (10).

Moreover, according to the current research by Damaskos et al. 2017, histone deacetylase inhibitors have a very promising potential to be used against breast cancer (11). This study also suggested transformed cells are more sensitive to Vorinostat while normal cells are relatively resistant to it. Also, Damaskos et al. 2017, proposed that the accelerated reversal of the binding of the HDACi to its target could accommodate normal cells with the ability to compensate for the inhibitory effects of these agents, while cancer cells with numerous deformities altering proteins regulating cell proliferation, survival, death, and migration are less like to have the ability to indemnify for the effect of the HDACi (11).

Future Research

The roles that HDAC's play in osteoclastogenesis suggest that they have potentially therapeutic potential for treating cancers, bone disorders such as osteoporosis and Paget's disease as well as HIV/AIDS and asthma.

HDAC therapy has a potential to become advantageous in orthodontics, since orthodontic teeth movement is strongly influenced

by remodeling, resorption and apposition of the bone. Furthermore, HDAC therapy could possibly be used for accelerated tooth movement, which has become a topic that brought a lot of interest and controversy to the orthodontic society around the globe (17).

Since there is not much information currently available on HDACs and HDAC Inhibitors, there are a lot more studies to be performed in order to understand the properties of HDAC and HDAC Inhibitors and how to expand the therapeutic use of them. For the future exploration of HDAC10, a series of over expression experiments could be performed in order to see the effects of HDAC10 on osteoclastogenesis when HDAC10 is over expressed.

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